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PREVALENCE OF CRYPTOSPORIDIUM OOCYSTS IN BOVINE AT DIFFERENT LIVESTOCK FARMS BY CONVENTIONAL MICROSCOPIC AND MOLECULAR TECHNIQUES

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ABSTRACT

Aim of the study was to analyze the prevalence of Cryptosporidiosis in cattle and buffaloes by oocyst microscopic examination and polymerase chain reaction (PCR). Cryptosporidiosis as detected by microscopic examinations was higher in cattle (10.5%) than buffaloes (8.47%) in Lahore from August, 2007-July, 2008. Percent prevalence of cryptosporidiosis in cattle and buffaloes was higher at Governmental dairy farms (20.55 & 16. 66) followed by Gawala colonies (12.77 & 9.44), Military dairy farm (6.11 & 4.44) and House hold dairies (3.88 & 3.34). The highest percent prevalence recorded in cattle and buffaloes was during summer (15 & 12), followed by autumn (10.88 & 20), spring (10.88 & 7.5) and the lowest in winter season (6.6 & 4.5). Infection rate was higher in young cattle and buffaloes than older. Non-significant difference was recorded in relation to sex of animals and infection rate. Percent prevalence in cattle (12.22%), as detected by PCR was highest at Governmental dairy farm (22.7%), followed by Gawala colonies (14.41%), Military dairy farm (7.7%) and House hold dairies (5%). Although, the prevalence of Cryptosporidiosis detected by PCR was higher as compared to microscopic examinations, no statistically significant difference was observed. It is concluded that cryptosporidiosis is highly prevalent in Lahore, which insinuates authorities for its monitoring by microscopic or molecular methods to achieve the production to its maximum potential.

Key words: Prevalence, Cryptosporidiosis, PCR, age, sex and season.

INTRODUCTION

Pakistan is growing in the field of livestock and facing a lot of infectious as well as non-infectious threats. Most of the livestock is not producing to the highest enough as in developed countries. Major involvement for this lowered performance is of different parasitic infections. These infections are causing huge economic losses to livestock industry in Pakistan. Cryptosporidium, a unicellular intestinal coccidian, infects microvillus of gastrointestinal tract in wide range of hosts including man (Spano *et al.,* 1998). Clinical disease is characterized by mucous to haemorrhagic diarrhoea, lethargy, pyrexia and loss of condition (Santin, 2013). Mortality by cryptosporidiosis may reach up to 35 percent in calves (Singh *et al.,* 2006). Contaminated environment surrounding the infected animal herds is a constant threat to healthy animals and human beings (Fayer *et al.,* 2012). Immuno-compromised individuals residing in close association with infected animals are more prone to infection (Semenza and Nichols, 2007). The infection persists until the immune response of the animal eliminates the parasite. This parasite can cause infection even on entry of few oocysts (Pantenburg *et al.,* 1995). Outbreaks of Cryptosporidiosis have been recorded in children by molecular technique (Helmy *et al.,* 2013).

Cryptosporidium parvum is major specie having zoonotic potential. However, other species including *Cryptosporidium bovis, Cryptosporidium andersoni and Cryptosporidium ryanae* have been isolated from infected cattle (Fayer *et al.,* 2006; 2008). Infection rate by parasites in cattle differ with growing age. Diagnosis of Cryptosporidium by the conventional microscopic methods such as fecal concentration techniques (zinc sulphate floatation and centrifugation) is a reliable indicator for presence of disease. Mostly identification is based on oocyst morphology (Zorana *et al.,* 2001) and immunoflourescence microcsopy (Atwill *et al*., 1999). Labelled antibodies against oocyst wall antigen have been used for identification. Wall antigens are common among many species and are not thought to be reliable (Fayer *et al.,* 2000). However, need is there to develop specific and sensitive detection for accurate diagnosis of etiological agents. Molecular technique especially Polymerase Chain Reaction (PCR) is highly specific and sensitive tool for accurate identification of Cryptosporidium species (McGlade *et al.,* 2003). Prevalence of Cryptosporidiosis in cattle and buffaloes reared under different management was determined in relation to month/season of the year, gender and age by conventional microscopy and PCR. So, dire need is there to optimize molecular detection technique for effective diagnosis in shortest possible time.

MATERIALS AND METHODS

Cryptosporidiosis, a common parasitic infestation, was assessed in cattle and buffaloes in relation to age groups, sex and seasons of the year at different dairy farms. Oocysts were identified on the basis of microscopic morphological features and confirmed by polymerase chain reaction (PCR), a molecular technique.

Experimental sampling: Fecal samples (N=1460) collected from cattle (N=720) and buffaloes (N=720) reared at Military dairy farm (Lahore Cantt), Government dairy farm (Pattoki and Dera Rakh Chahl), Gawala colonies (Canal road, Lahore) and House hold dairies (Bakar mandi) during August, 2007 to July, 2008. At Military dairy farm total numbers of cattle and buffaloes were 2410 and 312. In case of Government dairy farms buffaloes at Pattoki were 1103 and cattle at Dera rakh chahl were 210. Cattle and buffaloes included from Gawala colonies were 650 and 800. Total animals at selected House hold dairies included 900 cattle and 750 buffaloes.

Management of the animals was better at House hold dairies and Military dairy farm than Government dairy farms and Gawala colonies. At Military dairy farm animal sheds were clean, open, well ventilated with cemented floors and comparable in relation to hygiene with House hold dairies. Hygiene status was poor at Government cattle farm and Gawala colonies. Feed offered to animals at all of the selected farms consisted green fodder, wheat straw and concentrates. Quantity of concentrates offered was highest at House hold dairies followed by Military dairy farm, Government farms and Gawala colonies. Forty fecal samples from cattle and buffaloes (20 each) were collected from each selected dairy farm per month in sterilized properly labeled polythene bags and transported to postgraduate laboratory, Department of Parasitology, University of Veterinary and Animal Sciences, Lahore. Data on temperature, humidity and rainfall during the study period was noted to observe their relation with parasite infection rate (table 01).

Information regarding age, gender, collection date and name of selected farm was recorded on questionnaire. In both cattle and buffaloes, two age groups were specified i.e. younger (2-3 years) and older (3-7 years). Numbers of samples collected from young and old cattle/buffaloes were 180 and 540, respectively.

Microscopic identification of oocysts: Each of the collected fecal samples was processed for isolation of Cryptosporidium oocysts following the protocol described by Bairami Kuzehkanan *et al.* (2011). Zeihl Neelson's staining technique was opted as described by Henriksen and Pohlenz (1981). Prepared slides were compared with positive oocysts and identified on the basis of microscopic morphological features (Lindsay *et al.*, 2000).

Molecular detection: Cryptosporidium oocystes were identified at molecular level using polymerase chain reaction (PCR) as described by Johnson *et al.* (1995). Extraction of de-oxy ribose nucleic acid (DNA) was carried out following the method of da Silva *et al.* (1999) with minor modification. Instead of using FP 120 Fast Prep cell disrupter (Q-Biogene, Carlsbad. Calif.) for disruption of the Cryptosporidium oocysts tissue DNA extraction kit (GFC vivantis USA**)** was used. Amplification of targeted gene of Cryptosporidium oocyst DNA was obtained following the procedure described by da Silva *et al.* (1999). The primer sequences documented by Johnson *et al.* (1995) and da Silva *et al.* (1999) were used for PCR. The reference primers blasted through the program of bioinformatics and were found similar to the Cryptosporidium sequences. The 18s rRNA gene of parasite was targeted. The sequences of forward and reverse primers used were 5-AAGCTCGTAGTTGGATT TCTG-3 with synth ID No. 265261 and 5- TAAGGTGCT GAAGGAGTAAGG-3 with synth ID No. 265263, respectively. Amplified Cryptosporidium oocyst DNA was detected by horizontal agarose gel electrophoresis using 0.8% gel. Data was tabulated and analyzed by chi square test.

RESULTS AND DISCUSSION

Cryptosporidiosis is an endemic parasitic infection of ruminants as well as human beings in Pakistan. Cryptosporidium oocysts were identified on the basis of microscopic morphological characteristic and polymerase chain reaction (PCR). Prevalence of Cryptosporidium in cattle and buffaloes was determined in relation to animal age groups, sex and season at different livestock farms. Molecular technique was compared with usual conventional identification.

Prevalence of Cryptosporidiosis: Prevalence of Cryptosporidiosis in selected cattle carried out on the basis of oocyst microscopic morphology using Ziehl-Neelsen's staining technique. Similarly, Park *et al.* (2006) determined the presence of oocysts by light microscopy. Maurya *et al.* (2013) used modified Ziehl-Neelsen staining for screening of Cryptosporidiosis oocysts in fecal samples of cattle calves, buffalo calves, and lambs collected from selected states of India. However, morphological characterization of Cryptosporidium oocysts for identification of species is insufficient for epidemiological investigations. Molecular techniques are considered more reliable for confirmed reporting (Fayer *et al.*, 2006).

Percent prevalence of Cryptosporidiosis recorded was higher in cattle (10.5) than buffaloes (8.47) at Lahore from August, 2007-July, 2008. Statistically non-significant difference was observed in prevalence rate of Cryptosporidium oocyst between buffaloes and cattle by chi square test. The highest prevalence recorded for cattle

was at Government dairy farm (20.55%) followed by Gawala colonies (12.77%), Military dairy farm (6.11%) and the lowest at House hold dairies (3.88%). Similarly, in buffaloes highest percent prevalence was at Government dairy farm (16.66) followed by Gawala colonies (9.44), Military dairy farm (4.44) and the lowest at House hold dairies (3.34). On statistical analysis non-significant difference was observed in prevalence between Military dairy farm and House hold dairies whereas significant with other two farms. Percent prevalence at House hold dairies was lowest indicating better management and good feeding practices and differed non-significantly with Military dairy farm. Low rate of infection may be due to better hygiene status of these farms. Higher infection level at Gawala colonies and Government dairy farms is attributed to low leveled feeding and poor management.

Environmental factors play key role in the distribution of infection, rise and fall of oocyst counts in feces. Comparable observations were recorded by Park *et al.* (2006) in relation to prevalence in individuals having residences at varied environment. The rate of infection recorded on Oenarodo, Naenarodo and Nakwoldo islands was 6.0, 5.6 and 5.4 percents, respectively. Chai *et al.* (1996) observed similar pattern of difference in prevalence rate of Cryptosporidiosis on islands of Jeollanam-do and highest percentage recorded was 10.6. However, Yu *et al.* (2004) reported little lowered rate of infection (8.2%) at same locality. In agreement, Maurya *et al*. (2013) declared lower rate of infection in animals reared at range areas than dairies. So, rate of infection with Cryptosporidium differ under different surrounding environmental conditions both in animals and human beings.

Percent prevalence of Cryptosporidiosis in younger cattle was higher (15.5) than older (9.8). Both age groups differed significantly on statistical analysis by chi square test. Similarly, in buffaloes prevalence recorded was higher in younger (13.8) than older (9.8) and significant difference observed statistically. Cryptosporidiosis percent prevalence recorded in male cattle was little higher (11.25) than female (10.4). On statistical analysis by chi square test non-significant difference was noted. Cryptosporidiosis percent prevalence recorded in female buffaloes was higher (13.3) than male buffaloes (8.3).

In corroboration Starkey *el al.* (2006) documented 20 percent prevalence in young animals. Same pattern of infection in relation to age was observed by Santin *et al.* (2004). Atwill *et al.* (1998) reported higher prevalence at those farms having more number of young animals than others with low. The reduction in prevalence of Cryptosporidium oocysts with increasing age of animals was reported by Sturdee *et al.* (2003). Prevalence of Cryptosporidiosis observed by McAllister *et al.* (2005) in cows was 10.6-18.4 percent which is comparable with present findings. Close association between age of animal and infection with Cryptosporidium species was observed. Infection rate decreased with increase in age of the animals. This might be due to the development of immunity against the parasite. So, immune resistance with growing age and shifting of animals from highly polluted environment to lean one can reduce the load of infection by such parasites. In accord findings have been reported by McDonald *et al.* (2013).

Overall the highest percent prevalence of Cryptosporidiosis in cattle recorded was during summer (15), followed by spring/autumn (10.88) and the lowest in winter (6.6%). Prevalence during summer differed significantly on statistical analysis with other seasons. Non-significant difference ($P>0.05$) was noted between spring and autumn season whereas significant with winter. The highest percent prevalence of Cryptosporidiosis recorded in buffaloes was during summer (12) followed by autumn (20), spring (7.5) and the lowest in winter season (4.5). On statistical analysis prevalence was significantly higher ($P<0.05$) during summer/autumn than spring/winter. Results for monthly prevalence of Cryptosporidium in cattle and buffaloes are presented as figures (01&02).

Monthly distribution pattern of Cryptosporidiosis in bovine was related to the humidity, temperature and the rainfall. Percent prevalence was highest in the month of August. It was observed that high temperature (31.5^0C), humidity (71.3%) and rainfall (3.2mm) play role in higher loads of the parasite in domesticated animals. During the months of November, December and January temperature recorded was low (14.66°C) and humidity was 67.09 and 65.32 percent, respectively and prevalence was also very low. During summer rainfall was comparatively more in the year of study leading to humid environment. Much higher infection rates might be due to the favorable environment for parasitic infection. In agreement findings were reported by Donovan *et al.* (1998) that the prevalence at animal and farm level was highest in summer and dropped to lowest value in winter. Cryptosporidium infections were more frequent during rainy season in diarrheic patient at Bangladesh where as at Saudi Arabia high prevalence was recorded during autumn season in human beings (Donovan *et al.*, 1998). Maurya *et al.* (2013) documented that seasons have great impact on the rate of infection in animals and human beings. Katsumata *et al.* (1998) declared thick stocking density, rainfall, flood and contact with cats as significant risk factors.

Molecular identification of *Cryptosporidium* oocysts: Cryptosporidium oocysts in fecal sample collected from selected dairy farms were characterized using Polymerize Chain Reaction (PCR), a molecular technique. Amplified targeted nucleic acid revealed DNA band of 654 base pairs (Fig. 03). PCR based identification of Cryptosporidiosis was more sensitive than microscopic and is in agreement with the findings of McGlade *et al.* (2003) and Mathis *et al.* (1996). In total out of 720 extracted nucleic acid samples 88

amplified through PCR were declared positive. Molecular percent prevalence rate determined was 12.22 in cattle. Percent prevalence recorded using PCR was the highest at Government dairy farm (22.7), followed by Gawala colonies (14.41), Military dairy farm (7.7) and the lowest at House hold dairies (5). On statistical analysis infection rate on Government dairy farms differed statistically with other. Non-significant difference was recorded between Military dairy farm and House hold dairies whereas significant with other farms. Fayer *et al.* (2006) reported prevalence of Cryptosporidiosis in cattle vary at different farms under observation. At one farm prevalence noted was 3.4 percent whereas on other farm it was 28.6. Average infection rate detected by PCR at 14 farms collectively was 11.9 percent. Comparable results were observed by Santin *et al.* (2004) for Cryptosporidiosis in cattle using molecular technique.

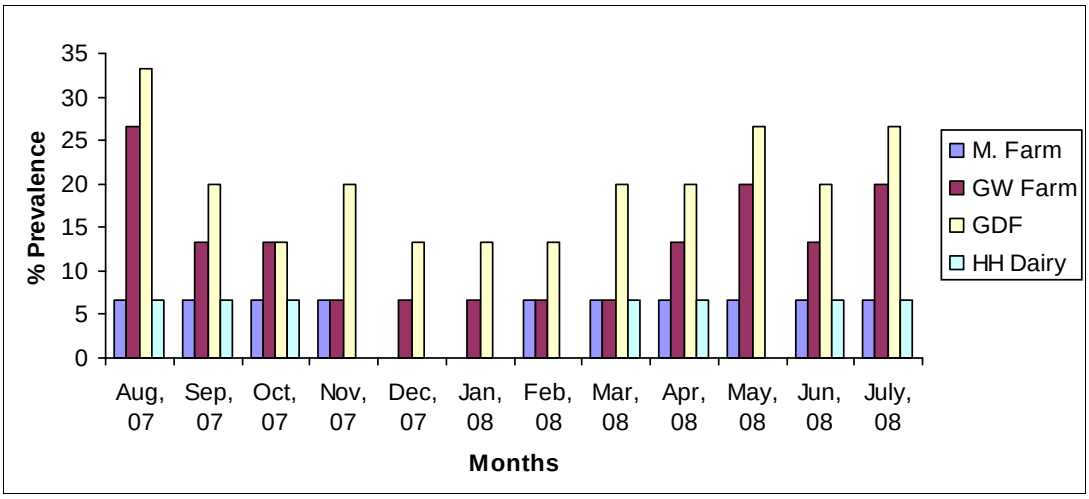

Fig. 01: Month wise percent prevalence of Cryptosporidiosis in Cattle at selected dairy farms of Lahore from August 2007 to July 2008

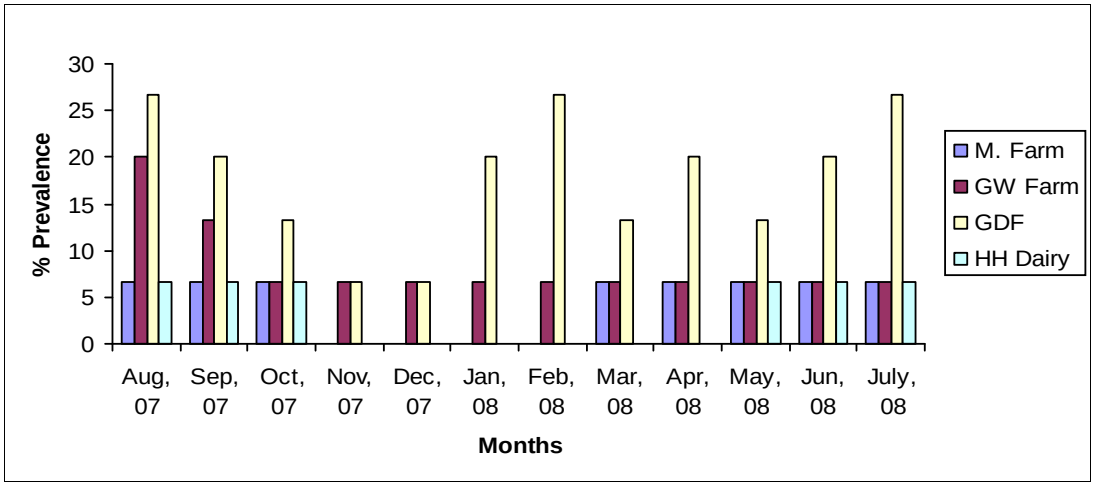

Fig. 02: Month wise percent prevalence of Cryptosporidiosis in Buffaloes at selected dairy farms of Lahore from August 2007 to July 2008

The highest season wise percent molecular prevalence was observed during summer (16.6) followed by autumn/spring (13.3), the lowest in winter (7.7). Prevalence of *Cryptosporidium* oocysts differed significant with other seasons. Difference between autumn and spring seasons was statistically non-significant whereas significant with other seasons. The higher molecular percent prevalence in young cattle (2-3 years) was higher (23.7) than those having age between 3-7 years (10.7). Statistically significant difference ($P<0.05$) was recorded between age groups. Molecular percent prevalence of Cryptosporidiosis in selected cattle was lower in females (13.6) than males (15). Statistically non-significant difference ($P<0.05$) was recorded on analysis of data by chi square test. Almost same pattern of Cryptosporidium infection rate was observed in cattle

reared under different management conditions. By microscopic examination percent prevalence recorded was 35.5 in 2004 whereas in 2002 and 2003 it was 50.3 and 19.7, respectively (Santin *et al.,* 2004). Xiao *et al.* (2004) declared 33.01 percent prevalence of Cryptosporidiosis on the basis of PCR and prevalence was higher in pre-weaned calves. Two peaks of higher infection were observed. First was of 66.7 percent at an age of less than two weeks and second (30.4%) at six months of age in calves. Huetink *et al.* (2001) reported comparable findings in relation to the peaks of infection associated with age. In present study prevalence was lowered with increase in age of the animals and higher at young age. Reduction observed in relation to age for Cryptosporidiosis had been documented by other workers in agreement to present study. Sischo *et al.* (2000) and Sturdee *et al.* (2003) observed comparable results in relation to age of the animals. Higher rates were recorded at early age than old animals. Most of the workers reported *Cryptosporidium parvum* infection at early age in cattle both by microscopy and PCR (Enemark *et al.,* 2002).

Difference in pattern of prevalence for identification by microscopy and molecular technique was in same fashion in relation to animal, age of animal, sex and season of the year. Statistically difference observed was non-significant. Slightly higher percentages were observed by PCR detection than identification on the basis of coprological examination.

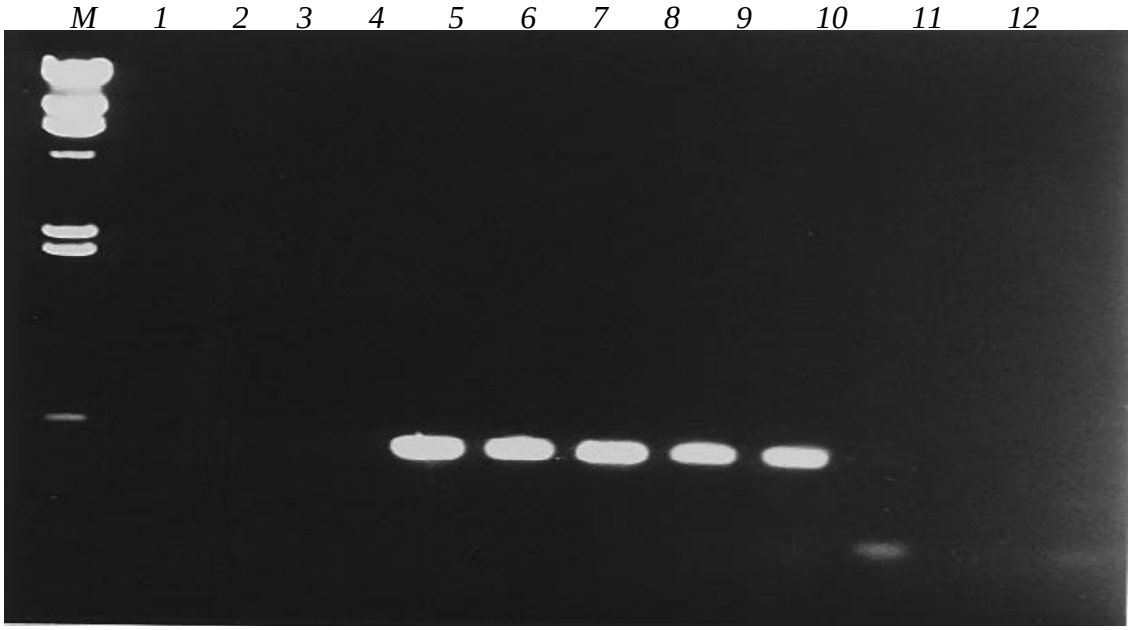

Fig. 03: Agarose gel electrophoresis pattern of PCR amplicons of *Cryptosporidium* oocysts

Lane M: 2 KB Ladder Marker, Lane 1, 2 & 3 Negative samples and Lane 4-9: Positive sample (654bp product)

Table 1. Mean month wise temperature, humidity and rain fall at Lahore during 2007-2008

Time (Months)	Temperature (îC)			Relative Humidity (%)			Rainfall (mm)
	Minimum	Maximum	Mean	Morning	Evening	Mean	
August, 2007	27.6	**35.37**	31.48	**76.19**	**66.38**	71.28	**3.2**
September,2007	25.4	**33.85**	29.62	**75.9**	**59.6**	67.3	**2.52**
October, 2007	19.20	32.50	25,85	67.37	85.08	76.22	0.00
November, 2007	14.44	26.39	20.41	81.23	52.96	67.09	0.34
December, 2007	08.50	20.83	14.66	81.20	49.45	65.32	0.106
January, 2008	06.09	17.80	11.94	76.29	46.32	61.30	0.78
February, 2008	09.40	21.17	15.28	72.65	47.53	60.09	0.19
March, 2008	19.10	30.90	**25**	67.30	36.80	52.05	0.00
April, 2008	28.38	33.30	30.84	53.13	33.10	43.11	1.93
May, 2008	25.40	37.70	31.55	47.36	31.70	39.53	1.18
June, 2008	27.15	35.82	31.48	72.34	55.20	63.77	2.38
July, 2008	28.36	35.49	31.92	77.35	62.48	69.91	3.78
Mean	19.98	30.09	25.03	70.77	52.46	61.61	1.45

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